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Tocopherol, phytosterol and phospholipid compositions of genetically modified peanut varieties

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Abstract: Genetic engineering offers great potential for developing peanut cultivars resistant to a broad spectrum of pathogens that pose a recurring threat to peanut health. In an effort to improve the disease resistance of peanuts, three transgenic peanut lines were developed. Somatic embryos of the peanut cultivar Okrun were transformed by inserting a chitinase gene from rice and/or a β-1-3-glucanase gene from alfalfa. The main objective of this research project was the assessment of composition of the nutritionally beneficial bioactive components in biotechnology-derived peanut lines. The transgenic peanut lines 188, 540 and 654, which showed increased resistance to fungal diseases, as compared to the parent line, were analyzed for their tocopherol, phytosterol and phospholipid compositions. The compositions of transgenic lines were compared to those of the parent cultivar. The experimental results indicate no major changes in the composition of transgenic peanut lines examined in this study with respect to the cultivar Okrun.

Keywords: phospholipid; phytosterol; tocopherol; transgenic peanut

INTRODUCTION

Fungal diseases of peanut, such as *Sclerotinia* blight, are a major problem limiting peanut production in Oklahoma. *Sclerotinia* blight (causal agent *Sclerotinia minor*) can cause yield losses of 10–50% depending upon the severity of infection. Development of disease resistance reduces use of chemicals for disease control. Genetic engineering of peanut for fungal resistance is a potential solution for the control of *S. minor*.

Chitinases are pathogenesis-related proteins.[1] They have been implicated in plant defense against fungal infection.[2] Hydrolases such as chitinase and β-1-3-glucanase are known to degrade the cell walls of many fungi that attack plants, making them rational candidates for over-expression through genetic engineering to produce disease-resistant crops.[3] The USDA-ARS Plant Science Research Laboratory in Stillwater, Oklahoma, focuses on developing alternatives to chemical pesticides to protect plants from insect and disease pests. The objective of their research is to develop transgenic lines of peanuts adaptable to the southwest with value-added characteristics and to reduce the impact of biotic and abiotic stress. Chenault *et al*.[3] have developed transgenic peanut plant lines containing antifungal genes from somatic embryos of the susceptible cultivar Okrun. Three transgenic lines, 188, 540, and 654, did show a significant increase in resistance to *S. minor* compared to the parent cultivar Okrun.[4,5]

Nuts are a good source of a wide range of nutrients and bioactive compounds with health benefits. Many of the bioactive compounds that occur in nuts are associated with the oil fraction. Nuts contain tocopherols, tocotrienols, phytosterols, phospholipids and many different flavonoids. There is scientific evidence that health benefits are mediated by the bioactive components in the oil fraction of nuts.[6] The effect of genetic engineering on these bioactive compounds is usually overlooked unless these components are the specific targets for modifications. However, these modifications may result in unintentional changes in the compositions of bioactive compounds naturally present in the plants. To our knowledge there is no comprehensive study on the composition of bioactive compounds in genetically modified peanut varieties. Hence, the main objective of this research project was to examine the compositions of bioactive compounds of transgenic peanut varieties. The specific objectives were as follows: (1) determination of phytosterol, tocopherol

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and phospholipid compositions of transgenic peanut varieties; (2) examination of the composition of their traditional counterpart; and (3) comparison of the compositions and determination of differences that might present safety or nutritional concerns or benefits.

EXPERIMENTAL

Variety selection

Okrun was released as a joint effort between USDA-ARS and Oklahoma State University.[7] Okrun originated from a cross of Florunner and Spanhoma and is commercially classified as a Runner variety. Okrun is susceptible to all the typical diseases that attack peanut.

Transgenic peanut lines, 188 and 540, were developed from Okrun somatic embryos and contain a chitinase gene from rice. Peanut line 654 is also a transgenic peanut line developed from Okrun somatic embryos and contains both a chitinase gene from rice and a β-1-3-glucanase gene from alfalfa. These transgenic peanut lines were tested for the presence of the transgenes by the polymerase chain reaction (PCR) and Southern blot and for transgene expression by colorimetric assays.[3] The peanut lines used in this study were grown in individual pots (10 cm diameter) in a mixture of sand, soil and shredded peat in a greenhouse. Healthy and mature pods were harvested for analyses. Progeny seed was again tested for the presence of transgene(s) via PCR.[3] The positive peanut seeds were placed in airtight plastic containers and stored in a freezer at $-20\,^{\circ}$C until further use.

Analytical methods

An accelerated solvent extraction unit (ASE300, Dionex Co., Sunnyvale, CA, USA) was employed for the extraction of oil from ground peanut seeds. A detailed description of the extraction system is reported elsewhere.[8] Three samples from each variety were used for oil extraction and chemical analysis. The tocopherol content of peanut oil samples was determined according to Katsanidis and Addis.[9] The individual tocopherol isomers were analyzed using a normal-phase HPLC column, Zorbax RX-SIL (5 μm particle size, 4.6×250 mm, Agilent Technologies, Santa Clara, CA, USA). The HPLC system (Alliance 2690 Waters Corp., Milford, MA) consisted of a separations module (Model 2695), a photodiode array detector (PDA) (Model 2996) and a multi-wavelength fluorescence detector (FD) (Model 2475). Both detectors were purchased from Waters (Milford, MA, USA). The oil sample was dissolved in hexane (12.5 mg mL^{-1}) and filtered through a 0.2 μm filter (Iso-Disc filter, Supelco, Bellefonte, PA, USA). The fluorescence detector was set at 290 nm excitation wavelength and 400 nm emission wavelength. Identification and quantification of chromatographic peaks were made by comparison with the responses of α, β, γ and δ-tocopherol standards (CN Biosciences Inc., La Jolla, CA, USA). An external calibration curve was prepared for each tocopherol standard to calculate the amount of tocopherols present in the oil sample.

The phospholipid standards, phosphatidylethanolamine (PE), phosphatidylserine (PS) from bovine and phosphatidylcholine (PC) from plant were purchased from Matreya Inc. (Pleasant Gap, PA). Phosphatidic acid (PA) from egg and phosphatidylinositol (PI)-Na salt from plant were from Matreya Inc. (State college, PA, USA). All the solvents used in analysis were HPLC grade and were filtered using a GH Polypro (47 mm, 0.45 μm) hydrophilic polypropylene membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) before use in HPLC analysis. A normal-phase silica column, μ Porasil 10 μm (3.9 mm i.d. $\times$300 mm) from Waters (Milford, MA, USA) was used for analytical separation of compounds. The mobile phase consisted of the following solvent mixtures: (A) hexane:water:isopropyl alcohol (40:58:2); (B) hexane:water:isopropyl alcohol (40:50:10). The flow rate was 1.0 mL min^{-1}. The solvent gradient system was as follows: 100% A to 100% B in 7 min, then held at 100% B for 6 min followed by 100% B to 100% A in 1 min and finally held at 100% A for 2 min. The total run time was 25 min, including 9 min isocratic equilibration time before each injection. An evaporative light-scattering detector (ELSD) (Model 2000, Alltech Associates Inc., Deerfield, IL, USA) and a photodiode array detector (PDA) (Model 2996, Waters Milford, MA, USA) connected in series was used for the analysis. The ELSD set points were as follows: nitrogen flow rate 3.5 mL min^{-1} and drift tube temperature 80 °C. The column temperature was set at 30 °C. The oil samples were dissolved in chloroform and filtered through a 0.2 μm Iso-Disc filter (Supelco, Bellefonte, PA, USA) for further analysis. Identification and quantification of chromatographic peaks were based on external standard curves prepared for individual standards.

The individual phytosterol standards, stigmasterol (95% purity), β-sitosterol (97% purity) (Sigma-Aldrich Corporation, St Louis, MO, USA) and campesterol (Matreya Inc., Pleasant Gap, PA, USA) were used for peak identification. Cholesterol oleate (98% purity, Sigma-Aldrich Corporation, St Louis, MO, USA) was used as an internal standard and quantification of individual phytosterols. The analytical method used for phytosterol analysis consisted of two steps, including hydrolysis of the oil samples and silylation, as explained elsewhere.[10] Derivatized free sterols were determined by using a Hewlett Packard (HP) 6890 gas chromatograph (Wilmington, DE, USA) equipped with an HP 5973 network mass selective detector (Agilent Technologies, Palo Alto, CA, USA). The phytosterol compositions of the samples were identified by direct comparison of their chromatographic retention times and the mass spectra with those of the authentic compounds. The peaks were also confirmed with NIST/EPA/NIH Mass Spectral

Library (Version 2.0). The data collection and analysis were managed using an HP Chemstation (Enhanced Chemstation G1701DA Version D.00.00.38, Agilent Technologies, Palo Alto, CA, USA).

Statistical analysis

All extraction runs and analyses were carried out in triplicate and in randomized order with the mean values being reported. Analysis of variance (ANOVA) of the results was performed using the General Linear Model of SAS (software version 8.1, SAS Institute Inc., Cary, NC, USA). Multiple comparisons of the various means were carried out by least significant difference (LSD) test at $P = 0.05$.

RESULTS AND DISCUSSION

The genetically modified peanut (GMP) lines were rich in α- and γ-tocopherol (Table 1). Okrun, the parent line, and GMP 654 had the highest total tocopherol content: about $33.2 \times 10^{-2}\,g\,kg^{-1}$ oil or about $18 \times 10^{-2}\,g\,kg^{-1}$ seed. Total and all the individual tocopherol contents of GMP 188 were significantly lower ($P < 0.05$) than all the other peanut lines examined in this study. However, the differences in tocopherol content and composition of GMP and the parent lines were minimal. For the GMP lines studied, there was no apparent trend indicating that genetic modification of peanuts extensively affected the tocopherol content of the seeds.

The total phytosterol contents of the GMP lines were in the range $330–445 \times 10^{-2}\,g\,kg^{-1}$ (Table 2). The GMP line 540 had the highest total phytosterol content. The total phytosterol contents of GMP lines were not significantly different than that of the parent line. β-Sitosterol was the major phytosterol in all the GMP and parent lines and constituted over 90% of the total phytosterols. The GMP 188 had significantly lower β-sitosterol content than that of the other lines. Campesterol and stigmasterol were the other phytosterols present in all the peanut lines studied.

Table 1. Tocopherol composition ($\times 10^{-2}\,g\,kg^{-1}$) of genetically modified peanuts

Sample	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol
Okrun	18.34a	0.85a	9.43b	4.76b
188	11.97c	0.50c	4.40c	4.22d
540	16.56b	0.80b	9.97a	4.75c
654	18.22a	0.88a	9.31b	4.81a

Means in the same column with the same letter are not significantly different at $P > 0.05$.

Table 2. Phytosterols composition ($\times 10^{-2}\,g\,k^{-1}$) of genetically modified peanuts

Sample	β-Sitosterol	Campesterol	Stigmasterol
Okrun	390.65a	13.22ab	18.04ab
188	296.22b	12.87b	20.92a
540	415.25a	13.77ab	15.69b
654	370.45a	14.80a	21.85a

Means in the same column with the same letter are not significantly different at $P > 0.05$.

Table 3. Phospholipid composition ($\times 10^{-2}\,g\,kg^{-1}$) of genetically modified peanuts

Type	PC	PE	PA	PI	Total phospholipids (mg $100\,g^{-1}$)
Okrun	229.9b	126.0b	82.8d	185.7a	624.4b
188	167.4d	103.3c	136.4c	96.0c	503.1c
540	211.8c	133.0b	212.7a	140.9b	698.5a
654	258.4a	148.1a	156.6b	139.8b	703.0a

Means in the same column with the same letter are not significantly different at $P > 0.05$.
Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatic acid; PI, phosphatidylinositol.

The GMP lines examined in this study contained about $500–700 \times 10^{-2}\,g\,kg^{-1}$ total phospholipids (Table 3). About 30–38% of the total phospholipids present in peanut seeds was PC. Large variations were observed in PA and PI content of genetically modified peanut lines. All the GMP lines contained significantly higher amounts of PA and lower amounts of PI than those of the parent line Okrun. However, PA, PI, PC and PE contents of all GMP lines were within the range observed for breeding lines and the phospholipid composition reported for traditional peanut varieties.[10] Hence, the compositional deviations in the GMP lines from the parent line reported here may not necessarily be a result of genetic modifications on the peanut seeds.

CONCLUSION

Although there were some statistical differences in chemical composition among the GMP lines, these variations were within the range reported for traditional peanut varieties. This study indicates that for the peanut lines studied genetic modifications did not cause substantial unintentional changes in composition of nutritionally beneficial bioactive components of peanuts. However, stability of the chemical composition of GMP lines over time needs to be further studied.

REFERENCES

1 Yun DJ, D'Urzo MO, Abad L, Takeda S, Salzman R, Chen Z, Lee H, Hasegawa MP and Bressan RA, Novel osmotically induced antifungal chitinases and bacterial expression of an active recombinant isoform. *Plant Physiol* **111**:1219–1225 (1996).

2 Boller T, Gehri A, Mauch F and Vogeli UC, Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* **157**:22–31 (1983).

3 Chenault KD, Burns JA, Melouk HA and Payton ME, Hydrolase activity in transgenic peanut. *Peanut Sci* **29**:89–95 (2002).

4 Chenault KD, Melouk HA and Payton ME, Field reaction to Sclerotinia blight among transgenic peanut lines containing anti-fungal genes. *Crop Sci* **45**:511–515 (2005).
5 Chenault KD, Melouk HA and Payton ME, Greenhouse testing of transgenic peanut for resistance to *Sclerotinia minor*. *Peanut Sci* **30**:116–120 (2003).
6 Haumann BF, Peanuts finds niche in healthy diet. *Inform* **9**:746–752 (1998).
7 Banks DJ, Kirby JS and Sholar JR, Registration of 'Okrun' peanut. *Crop Sci* **29**:1574 (1989).
8 Dunford NT and Zhang M, Pressurized solvent extraction of wheat germ oil. *Food Res Int* **36**:905–909 (2003).
9 Katsanidis E and Addis PB, Novel HPLC analysis of tocopherols, tocotrienols and cholesterol in tissue. *Free Radical Biol Med* **27**:1137–1140 (1999).
10 Jonnala RS, Nutritional composition of peanuts (*Arachis hypogae* L.) as affected by conventional breeding and genetic modification. MS thesis, Oklahoma State University Stillwater, OK (2004).